

ORIGINAL ARTICLE

**EFFECT OF ASCORBIC ACID AND ALPHA TOCOPHEROL SUPPLEMENTATION ON ACUTE RESTRAINT STRESS INDUCED CHANGES IN TESTOSTERONE, CORTICOSTERONE AND NOR EPINEPHRINE LEVELS IN MALE SPRAGUE DAWLEY RATS**

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**Background:** Stress of various origins suppresses male reproductive functions through releasing stress hormones. Antioxidant like ascorbic acid (AA) and alpha tocopherol (AT) have been thought to protect the body against stress induced damage. Whether, these antioxidants confer protection against the stress induced increased levels of corticosterone and nor-epinephrine, and decreased testosterone secretion have been investigated in this study. **Methods:** This quasi experimental study was carried out at the Department of Physiology, Army Medical College Rawalpindi in collaboration with National Institute of Health, Islamabad during March to September 2009. Eighty male Sprague Dawley rats were divided into five groups with sixteen rats in each group. Group-I served as the control without stress while group-II was exposed to restraint stress for 6 hours, group-III was administered AA, group-IVAT and group-V was supplemented with both the antioxidants along with standard diet for one month. All antioxidant supplemented groups were exposed to restraint stress for 6 hours. Immediately after the stress episode, blood sample was obtained for the assay of serum testosterone, serum corticosterone by EIA and plasma nor-epinephrine levels by ELISA. Data were analyzed on SPSS-13 and *p*-value less than 0.05 was considered significant. **Results:** Acute restraint stress resulted in a statistically significant rise in corticosterone and nor-epinephrine levels and fall in serum testosterone levels. AA supplementation for one month revealed insignificant changes in stress induced hormonal parameters. AT alone and in combination with ascorbic acid prevented the fall in testosterone level as well as rise in corticosterone, however nor-epinephrine levels remained unchanged. **Conclusion:** Supplementation with AT alone or in combination with AA prevent reduction in testosterone and rise in corticosterone levels while keeping the nor-epinephrine levels unchanged after acute restraint stress in Sprague Dawley rats.

**Keywords:** Acute restraint stress, testosterone, corticosterone, nor-epinephrine, ascorbic acid, alpha tocopherol

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**INTRODUCTION**

Stress is a proliferating problem of today's modern civilization where stressors are increasingly present.<sup>1</sup> Now a days, swift industrial progress, ecological contamination, rehabilitated lifestyles along with unfavourable employment situations are causative agents of stress in people.<sup>2</sup>

The function of stress hormones is believed to preserve homeostasis but these hormones may turn out to be detrimental if these are in excess or persist in blood, when no more required. As a result of this, these are likely to take part in the pathophysiology of many diseases.<sup>3</sup> Stress of various origin suppresses male reproductive functions.<sup>4</sup> Animal studies have shown that acute and chronic immobilization stress, result in decreased androgen levels. In humans, severe psychological stress due to death of a relative or spouse lowers sperm count which is most likely caused by stress induced decline in testosterone.<sup>5</sup>

Predominant glucocorticoid in rodents, like rats are corticosterone. Corticosterone levels are increased in psychological and physical stress, as in experimentally induced restraint stress in rats. Therefore, levels of serum corticosterone may serve as an appropriate indicator of stress in rats.<sup>6</sup>

Stress activates sympathetic-adrenal system with release of catecholamines like epinephrine and norepinephrine along with proportionate release of glucocorticoids in response to stress. Consequently, greater the glucocorticoids, more will be the norepinephrine levels.<sup>7</sup> Therefore, it would be of worth to measure the levels of norepinephrine to evaluate the stress induced changes.

Stress is generally thought to generate reactive oxygen species (ROS).When ROS surpass body's natural antioxidant defence, impairment to macro molecules such as DNA, proteins and lipids would occur. During stress, lipid peroxidation is increased in the body. As one of the eminent products

of lipid peroxidation is malondialdehyde (MDA), therefore, MDA is considered as the indicator of stress induced damage in terms of lipid peroxidation.

Antioxidants; ascorbic acid and alpha tocopherol are thought to protect the body against stress induced damage.<sup>8</sup> This study was therefore designed to determine the effect of antioxidants on stress hormones levels after exposure to the acute restraint stress. In addition, we investigated the preventive role of ascorbic acid and alpha tocopherol, on stress induced changes in corticosterone, norepinephrine, and testosterone.

## MATERIAL AND METHODS

It was a quasi-experimental study, conducted from March to October 2009 at Department of Physiology, Army Medical College Rawalpindi in collaboration with National Institute of Health (NIH) Islamabad. Healthy male Sprague Dawley healthy rats 90 days old, bred at NIH were obtained. Average weight of each rat was 275±50 grams. Animal house facility of NIH had the setup according to international standards for breeding and housing of research animals. The room temperature of animal house was maintained at 22±3 °C, with the help of central temperature regulating system.

Animals were placed in each cage of 2×3 feet size. Clean water bottles specific to fit over these cages for continuous supply of water were used. Rat feed was provided by the animal house of NIH according to international standards. Eighty rats were grouped into five; that is, 16 rats in each group. Each cage was labelled both for the group number and type of diet.

**Group-I** (n=16); served as the control group which was not exposed to restraint stress.

**Group-II** (n=16); Rats were exposed to acute restraint stress in a mesh wire restrainer for 6 hours. The rats of group I and II were fed normal standard non-supplemented diet along with plain tap water *ad libitum*.

**Group-III** (n=16); Rats were fed normal standard diet and ascorbic acid supplementation was given (500 mg ascorbic acid/l drinking water) for one month.

**Group-IV** (n=16); Rats were fed supplemented diet with 300 mg alpha tocopherol/kg chow mixed with 2% soybean oil for one month. These rats were given plain tap water *ad libitum*.

**Group-V** (n=16); Rats were fed supplemented diet with 300 mg alpha tocopherol/kg chow mixed with 2% soya bean oil and ascorbic acid supplementation as 500 mg ascorbic acid/l drinking water. At the end of 4 weeks of antioxidant supplement feeding, the rats of group III, IV and V were exposed once to

acute restraint stress in a mesh wire restrainer for 6 hours.

After that, the blood samples were collected, allowed to clot, centrifuged and serum was stored at -80°C till analysis. One ml of blood was transferred into EDTA container and centrifuged to obtain plasma. The analysis of serum testosterone, serum corticosterone was done by EIA and plasma norepinephrine by enzyme linked immune-sorbent assay (ELISA).

Data were analyzed on SPSS-13. The arithmetic mean and standard error of mean for all the groups were calculated. Difference in mean among the control and treated groups were calculated by the 'Independent sample *t*-test'. The difference was considered significant if *p* value was found to be less than 0.05.

## RESULTS

All animals in the study were monitored for their feed and well-being and found healthy and active throughout the study period.

Acute restraint stress resulted in a statistically significant rise in serum corticosterone ( $p<0.01$ ) and plasma norepinephrine ( $p<0.01$ ) and fall in serum testosterone ( $p<0.001$ ) levels as presented in Table-1. Ascorbic acid supplementation for one month showed insignificant change in stress induced alterations in hormonal levels (Table-2). On the other hand, one month supplementation with alpha tocopherol prevented significant fall in serum testosterone ( $p<0.001$ ) and manifested the rise in corticosterone level ( $p<0.01$ ) as shown in the Table-3. However, exposure to acute restraint did not alter the levels of norepinephrine ( $p>0.05$ ) in alpha tocopherol supplemented group (Table-3).

Similarly, the fall in testosterone and rise of corticosterone was significantly prevented ( $p<0.001$  and  $p<0.01$ ) respectively in the group supplemented with combination of ascorbic acid and alpha tocopherol (Table-4). However, levels of norepinephrine remained unchanged ( $p>0.05$ ) in the group of rats supplemented with combination of ascorbic acid and alpha tocopherol.

**Table-1: Effect of six hours acute restraint stress on serum testosterone, serum corticosterone and plasma nor epinephrine in male Sprague Dawley rats**

Parameter	Group-I (n=16)	Group-II (n=16)	<i>p</i> -value
Serum Testosterone (ng/ml)	6.09±0.15	5.18±0.19	<0.01
Serum Corticosterone (pg/ml)	8870.00±175.35	9688.75±219.18	<0.01
Plasma Nor epinephrine (ng/ml)	2.59±0.18	5.06±0.31	<0.001

**Table-2: Effect of one month, ascorbic acid supplementation on changes in serum testosterone, serum corticosterone and plasma nor epinephrine levels in male Sprague Dawley rats exposed to six hours acute restraint stress.**

Parameter	Group-II (n=16)	Group-III (n=16)	p-value
Serum Testosterone (ng/ml)	5.18±0.19	5.29±0.20	>0.05
Serum Corticosterone (pg/ml)	9688.75±219.18	9229.38±240.95	>0.05
Plasma Nor epinephrine (ng/ml)	5.06±0.31	5.45±0.21	>0.05

**Table-3: Effect of one month, alpha tocopherol supplementation on changes in serum testosterone, serum corticosterone and plasma nor epinephrine levels in male Sprague Dawley rats exposed to six hours restraint stress.**

Parameter	Group-II (n=16)	Group-IV (n=16)	p-value
Serum Testosterone (ng/ml)	5.18±0.19	6.80±0.23	<0.001
Serum Corticosterone (pg/ml)	9688.75±219.18	8710.63±149.15	<0.01
Plasma Nor epinephrine (ng/ml)	5.06±0.31	5.28±.17	>0.05

**Table-4: Effect of combined ascorbic acid supplementation and alpha tocopherol supplementation on changes in serum testosterone, serum corticosterone and plasma nor epinephrine levels in male Sprague Dawley rats exposed to six hours acute restraint stress.**

Parameter	Group-II (n=16)	Group-V (n=16)	p-value
Serum Testosterone (ng/ml)	5.18±0.19	7.06±0.15	<0.001
Serum Corticosterone (pg/ml)	9688.75±219.18	8776.25±157.71	<0.01
Plasma Nor epinephrine (ng/ml)	5.06±0.31	5.29±0.32	>0.05

## DISCUSSION

Exposure of Sprague Dawley rats to restraint stress for six hours resulted in rise in corticosterone and norepinephrine and a fall in testosterone. These findings are in accordance with the study conducted by Dong *et al.*, in which mice were exposed to the immobilization stress for three hours with resultant fall in testosterone and rise in corticosterone; the predominant steroid of rodents.<sup>9</sup>

The published literature support the concept that even two hours of immobilization stress to rats may decrease the testicular steroidogenesis along with an increase in the levels of serum corticosterone. It has been suggested that rise in corticosterone is responsible for the decline in testosterone.<sup>10</sup> Another study by Taylor *et al.*, points towards the fact that

immobilization stress causes a decline in testicular androgens.<sup>11</sup>

Restraint stress exposure to our experimental animals resulted in significant increase in the levels of norepinephrine. The rise in levels of catecholamines like norepinephrine is a well known finding in response to acute stress, which is the outcome of the stimulation of adrenal cortex and locus coeruleus.<sup>12</sup> Another study has indicated the increase in norepinephrine levels after cold induced stress.<sup>13</sup>

The antioxidants obtained from dietary sources, constitute the essential component of antioxidant defence system.<sup>14</sup> Many epidemiologic and clinical trials have reported that supplementation with antioxidant vitamins is associated with reduction in the incidence of chronic disease morbidity and mortality.<sup>15,16</sup> Therefore we investigated the effects of supplementation of two important, dietary antioxidants, i.e., ascorbic acid and alpha tocopherol. Our data show that the supplementation with ascorbic acid 500 mg/l drinking water for one month could not prevent the acute restraint stress induced rise in corticosterone and norepinephrine and decline in testosterone. Our previous study had shown that ascorbic acid supplementation does not affect the basal testosterone levels in rats.<sup>17</sup> Although numerous studies point towards the antioxidant role of ascorbic acid, but some studies did reveal that ascorbic acid may not ameliorate or prevent the effect of all stressors, like alloxan induced stress was increased by ascorbic acid supplementation.<sup>18</sup> Ascorbic acid is thought to prevent the oxidative stress induced derangements, like study by Ergul *et al.*, which narrates that ascorbic acid may reduce the oxidative damage to liver caused by anti-tuberculosis drug isoniazid.<sup>19</sup> This difference could be due to the difference in dosage of antioxidant, because Ergul and colleagues used the dose of ascorbic acid 100 mg/kg/day via oral route whereas we used the ascorbic acid as 500 mg/l drinking water. Therefore, it is proposed that ascorbic acid may prevent stress induced derangements at high doses.

Supplementation of the rats with alpha tocopherol for one month before the exposure to restraint stress significantly prevented the stress induced rise in corticosterone and fall in testosterone. Our results to assess the preventive role of antioxidant alpha tocopherol are supported by various international studies which have confirmed this role of alpha tocopherol. For example, administration of alpha tocopherol protects the rats against various stressors like homocystine induced oxidative stress.<sup>20</sup> In another study, intra peritoneal administration of alpha tocopherol in a dose of 50 mg/kg/day to streptozotacin induced diabetic rats significantly lowered the oxidative stress in the nervous tissue of rats.<sup>21</sup>

Combined supplementation of our experimental animals with ascorbic acid and alpha tocopherol for one month before exposure to restraint stress, significantly prevented the stress induced rise in corticosterone and fall in testosterone. The preventive role of ascorbic acid and alpha tocopherol supplementation against various types of stressors is well documented by various studies.<sup>22,23</sup>

Although some authors are of the opinion that combination of antioxidants have no added advantage as reported by Huang *et al.*, in which they had given antioxidants in combination to humans and their results revealed no added advantage of combined or alone use of anti-oxidants.<sup>24</sup> On the contrary, when ascorbic acid was added to the cultured hepatocytes, it prevented the loss of alpha tocopherol and increased its availability.<sup>25</sup>

Decline in testosterone levels in our stress exposed groups could be due to the marked increase in lipid peroxidation as narrated by data of our another study displaying increased malondialdehyde (MDA) levels in the groups exposed to stress.<sup>26</sup> It means, that the restraint stress may induce oxidative type of stress with resultant generation of reactive oxygen species (ROS).<sup>27</sup> ROS most commonly target polyunsaturated fatty acids of cell membranes with resultant lipid peroxidation caused a rise in MDA.<sup>28</sup> Rise in MDA after restraint stress, was prevented in the rats supplemented with alpha tocopherol and combination of alpha tocopherol and ascorbic acid as shown in data of our other study.<sup>26</sup> It means the lipid peroxidation decreased and the resultant membrane stabilizing effect could be responsible for the favourable preventive roles of these antioxidants. There is a need to investigate the effects of these antioxidants at Leydig cell levels to appraise their mechanism of action.

## CONCLUSION

Supplementation with alpha tocopherol alone or in combination with ascorbic acid prevent reduction in testosterone and rise in corticosterone levels while keeping the norepinephrine levels unchanged after acute restraint stress in Sprague Dawley rats.

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